In vivo Raman flow cytometry for real-time detection of carbon nanotube kinetics in lymph, blood, and tissues

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Abstract. Nanoparticles are intensively being explored as contrast agents for medical diagnostics and therapies using various optical methods. We present the first demonstration of the use of time-resolved Raman spectroscopy for in vivo real-time detection of circulating carbon nanotubes (CNTs) or cancer cells labeled with CNTs in the lymph, blood, and tissues of live animals with fast spectral acquisition times of down to few milliseconds. After intravenously administering CNTs in the tail vein of the rat, this technique provides the ability to detect the circulation of CNTs in the blood microvessels of the intact rat ear. The capability of Raman spectroscopy is also demonstrated to monitor, identify, and image the CNTs during their transportation by lymphatics in the rat ear and mesentery. The strong and specific Raman scattering properties of CNTs make it possible to detect in vitro and in vivo single cancer cells (HeLa) tagged with CNTs. In vivo Raman flow cytometry opens a new avenue for multiparameter analysis of circulating nanoparticles with strong Raman scattering properties and their pharmokinetics in blood and lymph systems. Moreover, this technology has the potential for molecular detection and identification of circulating tumor cells, and infections labeled with CNTs. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3119145]

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1 Introduction

Carbon nanotubes (CNTs) with unique, well-controlled chemical, physical, electronic, and mechanical properties exhibit great potential in biology and medicine in areas such as gene/drug delivery, bacterial filters, molecular diagnosis, and cancer and infection treatments. Before any clinical applications of CNTs can be practical, it is imperative to determine the critical in vivo parameters, namely, pharmacological profiles such as the CNT clearance rate from the blood circulation and their dynamic distribution in tissues and organs.

The first CNT-related studies were performed ex vivo in static conditions using blood sampling and radioactive or near-infrared (NIR) fluorescence techniques. The high NIR absorption of CNTs provided the opportunity to use them as excellent photoacoustic (PA) contrast agents for the detection of circulating cells and pathogens as well as tumor imaging. An alternative, Raman spectroscopy has been applied to monitor the CNTs in vivo in static condition, demonstrating large signal-to-noise ratios (SNRs) and molecular specificity due to the strong Raman scattering signals from CNTs and their specific vibrational spectra fingerprints, respectively. Raman spectroscopy was successfully used also in in vitro flow cytometry using surface-enhanced Raman scattering (SERS) nanoparticles. However, the application of Raman flow cytometry (RFC) for in vivo studies, especially for single cell detection, has not been reported. Here, we demonstrate the ability of CNTs to serve as excellent in vivo Raman contrast agents, resulting in a time-resolved measurement of the CNTs kinetics in blood and lymphatics, as well as selective detection of single cancer cells in small animal models. In particular, this paper presents the successful use of the in vivo RFC approach in two various applications: (1) in vivo real-time detection of single-walled CNT clearance in lymph and blood flow and (2) detection of single cancer cells labeled with CNTs in biological tissues.

2 Materials and Methods

2.1 CNT Preparation

Single-walled CNTs were grown by radio-frequency chemical vapor deposition (rf-CVD) on a Fe:Mo/MgO catalyst with methane as the carbon source and processed, as described...
previously. The individually dispersed CNT solution was obtained by wrapping double stranded DNA around the nanotubes, an approach that was used previously to individually disperse CNTs in liquid solutions. Salmon double-stranded deoxyribonucleic acid (dsDNA) purchased from Sigma-Aldrich was introduced in the buffer solution (10 mM, pH = 7.3) at a concentration of 0.7 mg/mL. Then 0.4 mg of CNTs were added into 5 mL of dsDNA buffer solution followed by a strong sonication over a water-ice bath (Branson Model 1510R-MT, 42 kHz, output power 70 W) for 1 h. The resulting solution was centrifuged for 15 h at 16,000 g to remove the undispersed CNTs. The result was a uniformly darker solution with no visible agglomerations of CNTs. This approach can be used as a preliminary step toward the complete biocongjigation of CNTs to antibodies and folates for controlled delivery of CNTs to tumors.

2.2 CNTs Characterization

The CNT physical characteristics after their synthesis were assessed using several analytical techniques that included microscopy, spectroscopy, and thermogravimetric analysis.

2.2.1 Atomic force and transmission electron microscopy

The CNT length analysis and their individual dispersion were studied by atomic force microscopy (Veeco Multimode Scanning Probe Microscope with Nanoscope IIIa Controller; Veeco Instruments, Woodbury, New York) and transmission electron microscopy (TEM) using a JEOL 2100 FE electron microscope (JEOL-USA, Peabody, Massachusetts). For TEM analysis, the CNTs were deposited onto carbon-coated copper grids without staining to reduce the possibility of artifacts.

2.2.2 Epifluorescence and light microscopy

Phase-contrast and transmittance microscopy was performed using a light microscope (Axioplan 2 Plus, Carl Zeiss, Inc., Germany) equipped with a 12-bit Color MicroImager II Cooled digital camera (QImaging, Burnaby, Canada) with a resolution of 1.3 million pixels. The 100X or 63X oil immersion objectives (Carl Zeiss) were used to visualize and acquire the images and their sequences with a digital image and video recording software, StreamPix (Norpix, Inc., Montreal, Canada). The light microscopy system was additionally equipped with a filter set (FITC, Carl Zeiss) consisting of a bandpass filter covering 450 to 490 nm for an exciter and an absorbance filter covering a wavelength of 515 nm to acquire epifluorescence images for the live (green fluorescent) and dead (red fluorescent) cells (see later). Fluorescent dye—FITC-dextran 150,000 (Sigma Chemicals)—was used for the visualization of lymphatics and verification of Raman data.

2.3 Confocal Raman Microscope/Spectrometer

For the in vitro and in vivo experiments, three lasers in continuous wave (cw) mode were alternatively used for the excitation of Raman spectra: (1) argon laser with a wavelength of 514 nm and power from 2 to 99 mW; (2) a He-Ne laser with a wavelength of 633 nm and power from 5 to 20 mW; and (3) a diode laser with a wavelength of 785 nm and power from 5 to 99 mW. The scattered light was collected in the back-scattering geometry by a confocal Raman microscope on an Olympus BX-51 platform, with 10, 50, and 100× microobjectives. These microobjectives were simultaneously used to focus the incident beam onto the sample. The adjustable laser circular beam size ranged from 1 to 10 μm. An automated stage provided 3-D (X-Y-Z) spatial scanning with a step size of 1 μm. Raman scattering spectra were obtained with a Raman spectrometer (Horiba Jobin Yvon LabRam HR800) with 1800- and 600-lines/mm gratings equipped and a Peltier-cooled CCD camera. The spectral resolution was below 0.3 cm⁻¹, and in most experiments the collected signal for each data point was the average of 10 measurements. Raman spectra were calibrated with a standard silicon wafer using a specific peak of 520 cm⁻¹, corresponding to the Si-Si stretching band.

2.4 Cell Cultures

For the primary culture the frozen cells (Human Cervical cancer HeLa Cells) were thawed and seeded in 75-mm² flasks at a density of 1 × 10⁶ cells, harvested under minimum essential medium (MEM), and supplemented by 10% FBS, 1% PS, and 1% glutamic acids (nonessential amino acid). The cells were cultured at 37 °C and 5% CO₂ for several days to grow and develop, while the medium was changed every second day. After the medium was carefully removed, the cells were treated with 1× trypsin in phosphate-buffered saline (PBS) and incubated for 10 min for detachment followed by centrifugation at 1100 rpm for 10 min. The supernatant was removed and the cells were resuspended into a fresh medium with dsDNA-wrapped CNTs. The CNT concentration was adjusted based on the desired experimental design, as presented. After the administration of CNTs, the cells were incubated in 75-mm² flasks with the CNT solution for 12, 24, and 48 h under identical conditions before the cells were used for experiments. For the cytotoxic studies, the cells were trypsinized and cultured in 35-mm culture plates (1 × 10⁵ cells/plate) with different concentrations of single-walled CNTs for 48 h under identical conditions. Standard ethidium bromide (EB) and acridine orange staining kits (1 part of EB in PBS: 1 part of 100 mg/ml of acridine orange in PBS) were used to detect and analyze the number of dead cells. At least 300 cells were examined under the fluorescent microscopic camera with a 10× objective. Acridine orange is a commonly used dye, which stains the live cells green while the dead cells are stained brown by the EB. To observe the cytotoxic effects of the CNTs only, all the cell viability results were normalized. A one-way analysis of variance (ANOVA) was used for the statistical analyses of the percentage of the dead cells; P values of 0.05 or less were considered to indicate significance.

2.5 Animal Model

The feasibility studies of in vivo RFC involved male Fisher (F344) rat models at ~4 weeks of age weighing 150 to 180 g. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas for Medical Sciences (UAMS). The rat mesentery was used for proof of concept in vivo RFC because it consists of a very thin (7- to 15-μm) transparent connective tissue with a single layer of blood and lymph microvessels. After administering standard anesthesia [ketamine/xylazine, 50/10 mg/kg, intramuscular (im)] the rats were laparoto-
mized by a midabdominal incision. Immediately after exposure, the mesentery was placed on a customized and heated module fixed on the automated stage of the Olympus microscope. The mesentery was suffused with warmed Ringer’s solution (37 °C, pH 7.4) containing 1% BSA to prevent protein loss. Besides mesentery, the vasculature was also examined in the intact rat ear. We studied the ear blood vessels were located 50 to 150 μm deep and had diameters in the range of 30 to 50 μm and blood velocities of 1 to 5 mm/sec. Lymph microvessels under study had a similar depth and 150 to 200 μm diameters with average lymph flow velocity in the range of 0.1 to 0.5 mm/s. The laser radiation was focused either on the interstitial ear tissue, blood or lymph microvessels, or in the mesentery vessels, depending on the experiment performed. The CNT solution was delivered with a 28 gauge needle inserted intravenously in the rat tail vein or skin. Additionally, cancer cells with CNTs were administered locally in the rat mesentery or injected in the ear tissue on areas close to lymph vessels.

2.6 Temperature Measurements

The thermal analysis was performed using a calibrated forward-looking IR (FLIR) Thermacam SC500 thermal camera (FLIR Systems, Inc., Boston, Massachussetts). The thermal images were acquired at 1 Hz and recorded to a sequence file. The camera lens used has a field of view (FOV) of approximately 4 X 6 mm with a maximum resolution of 18 μm/pixel. Images were recorded prior to and during the laser exposure of tissue.

3 Results

3.1 Carbon Nanotube Characterizations

Figure 1(a) shows experimental schematics of in vivo RFC for real-time monitoring of carbon nanotube biodistribution in a complex biological environment (e.g., in lymphatics) based on their strong Raman scattering and unique Raman spectra (b). (c) The spatial structure (left) and TEM image (right) of carbon nanotubes with diameters ranging between 1 and 2 nm; these nanoparticles in PBS solution were injected intravenously in the bloodstream or in skin. (d) Human cervical cancer HeLa cells were incubated with carbon nanotubes and injected in lymphatics or ear skin.
strongly depend on the diameters and chirality of the nanotubes and a high-wave-number region (tangential and longitudinal modes) between 1200 and 3000 cm\(^{-1}\) (Fig. 1(b)). The D band, positioned between 1250 and 1450 cm\(^{-1}\), is associated with vacancies and the presence of other carbonaceous impurities (amorphous carbon, glassy carbon, etc) that destroy the graphitic symmetry. Band G (between 1500 and 1600 cm\(^{-1}\)) corresponds to the splitting of the \(E_{2g}\) stretching mode for graphite. The 2-D band (between 2450 and 2650 cm\(^{-1}\)), which is the second-order harmonic of the D band, is associated with the degree of crystallinity of the graphitic layers. The G band has the highest intensity (and referred to as Raman signals) and therefore it was further used to dynamically evaluate the presence of CNTs in the biological environments in vivo.

### 3.2 Comparison of Excitation Wavelengths

Initial experiments using in vivo optical imaging and ex vivo immunochemistry assays revealed that laser radiations of 633 and 785 nm wavelengths at power levels of less than 20 mW did not produce any visible tissue and vessel photodamage. Laser radiation at 514 nm at the same power was found to induce lymph vessel constrictions and local tissue damages. In addition, higher blood absorption of the 514-nm laser radiation, compared to that of 633 or 785 nm, may restrict its application for studying tissues with rich blood networks. Raman signals collected with the 514-, 633-, and 785-nm laser radiations were found to be comparable in intensity (slightly higher for the 633-nm excitation), while the background signal levels were found to decrease as the excitation wavelength increased (Fig. 2). Thus, the laser radiations of 633 nm and especially 785 nm are the most promising for the CNTs in vivo detection since the background signals of the surrounding tissues have minimum absorption levels in the NIR spectral range. Temperature measurements of a 10-ml CNT solution (0.2 mg/mL) irradiated separately for 1 min with each of the three laser wavelengths (514, 633, and 785 nm) revealed a 4 to 5 °C temperature increase when exposed to the 785-nm laser when compared to lower temperature variations (less than 1 °C) for the 514- and 633-nm lasers. These results indicate a higher optical absorption, and hence, heating of CNTs when irradiated with the 785-nm laser, compared to when exposed to the 514- and 633-nm laser radiations. In this paper, a laser wavelength of 633 nm was selected as a reasonable compromise between Raman signal levels (strong enough for CNT detection), minimal thermal effects, and laser penetration into the tissue of a few millimeters, which is sufficient for the Raman assessment of peripheral vessels.

#### 3.2.1 In vitro identification of HeLa cancer cells labeled with CNTs among red blood cells

The increase of the incubation time (12, 24, to 48 h) of human cervical HeLa cells with the DNA-wrapped single-walled CNTs led to a gradual enhancement of the Raman signal intensity collected from areas inside the cells (Fig. 3(a)). These data indicate an increased CNT concentration and number of CNT clusters inside the cells, which were present mostly around the nucleus membrane (Fig. 3(b)). These results indicate a high accumulation of CNTs both in the cell cytoplasm and inside the nucleus, especially, at increased incubation times. The CNT translocation in vitro into cells most probably happened due to endocytosis and other complex processes such as diffusion through transmembrane channels, or adhesive interactions. Some of the most important factors that are responsible for the uptake of CNTs by the cancer cells include their size, surface charge, concentration, temperature, and the biochemical conditions of the delivery. CNT structural characteristics such as the diameter and length are the parameters responsible for their penetration into the cells since they present a snaking behavior when they cross the intracellular membranes. In our study, surprisingly, the CNTs were found to aggregate around the membrane of the nucleus and then penetrate the nuclear membrane into the nucleus after being dispersed individually in the medium solution that was used to feed the HeLa cells. The significant enhancement of the Raman signals from the CNT aggregates present inside the cells provides a direct path for highly sensitive Raman detection of these cells in different biological environments. This is analogous to the synergistic amplification of the PA and photothermal (PT) effects due to the self-assembled nanoclusters inside the living systems. Figure 3(b) at the bottom shows the capability of Raman spectroscopy to simultaneously detect and identify (using the specific CNT G band spectral position) a single HeLa cell with CNTs (48-h incubation) in a monolayer of red blood cells (RBCs) from a rat. The results were obtained in vitro by spatially scanning the laser beam across the sample with a velocity of 0.01 mm/s and “spectral” scanning (i.e., collection of a 1200- to 1800-cm\(^{-1}\) Raman spectral window) at a 50-ns acquisition time. Significant high SNRs and low background signal levels of the RBCs also enabled us to distinguish one cancer cell among hundreds of RBCs in a concentrated blood sample 120 μm thick (not shown here).

#### 3.2.2 In vivo detection of the carbon nanotubes distribution in rat ear tissue

To further estimate the in vivo maximal achievable sensitivity of the technique in almost ideal static conditions, a small amount of CNT solution (10 μL, concentration of 0.2 mg/ml) was locally injected under the skin of the rat ear in an area...
free of blood microvessels. The high-resolution transmission imaging of the ear tissue was used to visualize the location of the blood vessels as well as the lymphatic vessels networks highlighted by a local administration of conventional Evans Blue dye [Fig. 4(a)]. The optical monitoring of this area revealed that the initially highly localized CNTs [Fig. 4(b)] arrow evolved into an elongated zone about 20 min after delivery [Fig. 4(c)] arrow.

The collection of specific Raman spectra of the CNT-free ear tissue (e.g., before the CNT injection) required relatively long acquisition times because of the relatively low intensity of Raman signals. As an example, Fig. 4(d) shows representative Raman spectra (out of over 50) of the tissue collected in vivo at 30-, 40-, 50-, and 60-s acquisition times. Figure 4(d) indicates that even for relatively long acquisition times (over 60 s) the Raman spectra of the biological tissues did not show any peak at 1568 cm\(^{-1}\) [therefore specific only to CNTs—Fig. 1(b)] among the distinctive spectral features of the tissues.

One minute after the CNTs administration, the Raman signal started being collected from the tissue area with the maximum concentration of CNTs [Fig. 4(b)] at different acquisition time intervals and different wavelengths. The 1568-cm\(^{-1}\), CNT-specific peak was visible among the background Raman signals from the ear tissue even for acquisition times of as low as a few dozens milliseconds [Fig. 4(e)]. When the acquisition time was increased, the 1568-cm\(^{-1}\) peak became better defined and increased in intensity. At a 2-s acquisition time, besides the G band, the nanotubes’ D band (1312 cm\(^{-1}\)) also became evident in the Raman spectra [Fig. 4(e)]. These data clearly indicate the difference in the Raman scattering properties of CNTs and biological tissues, especially for short acquisition time intervals (<5 s) when there is a smooth background from tissue with no distinguished Raman spectral features [Fig. 4(e)] bottom. Note that the increase of the 1568-cm\(^{-1}\) peak intensity was not accompanied by a similar enhancement of the background signal collected from the surrounding tissue, thus leading to the enhancement of the SNR. Time-related Raman spectral monitoring of the ear tissue (with the acquisition time of 2 s) in an area close to the initial spot of high CNT concentration [see arrow in Figs. 4(b) and 4(c)] and with the starting analysis time of 10 min after CNT injection, demonstrated a gradual enhancement of the 1568-cm\(^{-1}\) peak intensity, indicating a continuous concentration increase of CNTs at the point of analysis due to their bioredistribution [Fig. 4(f)].

Taking into account a previous lymphatic study\(^2\), the obtained kinetic data strongly suggested that CNTs, after their migration into the tissue, were absorbed by the neighboring lymph vessels, followed by their further propagation by the lymphatics. Indeed, regular CNT tissue diffusion usually results in a relatively homogeneous CNT distribution around the injected area, while both the optical images and Raman analysis indicated a significant increase of CNT concentration in one dominant direction, which is likely associated with the lymph microvessels located in the area of CNT injection [Fig. 4(a)].

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3.2.3 \textit{In vivo Raman detection of circulating carbon nanotubes in rat ear blood vessels}

To determine the clearance rate of CNTs, the 100-μl PBS suspension of CNTs (0.2 mg/ml) was injected into the rat blood circulation through the tail vein followed by real-time monitoring of circulating CNTs in a 50 to 70-μm blood ear vessels [Fig. 4(e)] at fixed wavelength near the CNT G band (1568 cm\(^{-1}\)). The Raman signals quickly appeared within the first minutes, providing slightly fluctuated continuous background with the average Raman signal amplitudes exceeding 5 to 10 times the Raman background signals from blood vessels. Simultaneously, stronger but less frequent Raman signals were observed above relatively stable Raman backgrounds from CNTs as discussed before. These phenomena can be associated with the random fluctuations of the CNTs number in the detected volume (i.e., within the focal volume of the excitation laser beam) or appearance of small CNT aggregates. This study revealed the average clearance time of the CNTs from the rat ear microcirculation to be in the range of 20 to 30 min, depending on the quantity of solution and presence of CNT aggregates. No Raman signals of the 1568-cm\(^{-1}\) peak were observed 30 to 40 min after injection. The ob-

\[\text{Fig. 3} \quad \text{(a) In vitro Raman signals (bottom) (633-nm laser excitation, 20-mW power, and an acquisition time of 50 ms) from single He-La cells in culture petri dishes incubated for 12, 24 and 48 h with CNTs (top). (b) In} \]

\[\text{in vivo Raman spectra (bottom) of single He-La cells after 48 h incubation with CNTs that was introduced among a monolayer of red blood cells. (RBCs) (top); these data were obtained simultaneously by a fast spectral analysis with an acquisition time of 50 ms and relatively slow laser spatial scanning at a rate of 0.01 mm/s.}\]
served CNT clearance rate was found to be in a reasonable correlation with the values obtained when using fluorescent and radioactive labels \textit{ex vivo}.

3.2.4 \textit{In vivo} detection of cancer cells tagged with carbon nanotubes in rat ear tissue

Next we investigated the potential of RFC to detect cancer cells \textit{in vivo}. HeLa cells were incubated with CNTs for 48 h and injected in a similar manner as the CNTs in the rat ear tissue followed by Raman spectral and spatial scanning (633-nm laser excitation, 20-mW power, 0.5-s acquisition time) of the tissue near the injection site. The characteristic peaks of the CNTs are evident in the spectrum (with an intensity of around 150 counts) above background signals obtained before the administration of cells [Fig. 4(h)]. This high SNR enabled the collection of an \(X-Y\) Raman map based on the \(X-Y\) variation of the 1568-cm\(^{-1}\) peak intensity. The localized Raman signal 2-D distribution presented in Fig. 4(g) corresponds to the presence of only a single HeLa cell in the ear tissue as verified by (1) low concentration of the injected cells, (2) signal counting from individual cells before injection [Fig. 3(a) and 3(b)], comparability of signal dimension localization with single cell size (~30 \(\mu m\)).
3.2.5 In vivo Raman detection of carbon nanotubes and cancer cells in rat mesentery lymphatics

To further verify the previously presented finding of CNT uptaking by lymphatics, the CNT solution (0.05 mg/ml, 100 μl) was topically administered onto the rat mesentery—a thin and relatively transparent structure with clearly distinguished individual 100 to 200-μm-diam lymph vessels [Fig. 5(a)], placed on a heated microscope stage and bathed in warm Ringer’s solution 37 °C, pH 7.4. The laser beam was focused onto the area behind the lymph valve [Fig. 5(a)], which acts as a natural nozzle, concentrating the flowing CNTs near the vessel axis. Similar to the CNT blood flow experiments [Fig. 4(g)], time-resolved Raman monitoring of lymphatics was performed by analyzing the 1568-cm⁻¹ peak intensity, which is in direct relationship with the number of CNTs present in the focal volume of the laser beam. Taking into consideration the laser scattering phenomena in tissue, this volume was estimated to be approximately 20 to 30 μm³.

As shown in Fig. 5(b), the time required for the peak at 1568 cm⁻¹ to become present in the Raman spectra of the lymph vessel (i.e., time scale of most CNTs uptaking by lymphatics) at an acquisition time of 50 ms was approximately 3–5 min after the local administration of the CNTs. To avoid background Raman signals due to the CNTs possibly present on the mesentery surface, the surface of the area exposed to the laser radiation was continuously washed with warm Ringer’s solution. This procedure did not influence the Raman signal levels, suggesting the presence of CNTs only inside the lymph vessels. Raman data indicated that the intensity of the G band gradually increased with the measurement time, which showed a continuous enhancement of the CNT concentration in the lymph vessels due to the CNTs uptaking from the surrounding interstitial tissue. After 15 min, the CNT con-
The concentration inside the lymph vessel was found to decrease, as indicated by Fig. 5(b). CNT-specific Raman signals were detected even beyond 35 min after the initial administration, but were of a significantly lower intensity.

Identical experiments were repeated by the administration of HeLa cells tagged with CNTs (48-h incubation time) onto the mesentery. The presence of HeLa cells inside the mesentery was verified by optical transmission imaging [Fig. 5(c)]. While the presence of the CNTs in these cells was manifested by a significantly higher intensity of the 1568-cm\(^{-1}\) G peak (106 counts; acquisition time of 0.5 s) above the mesentery tissue background [Fig. 5(d)]. Finally, the consecutive Raman measurements at a 1-s time interval provided simultaneous detection and identification of a single HeLa cell through spatial, temporal, and spectral changes of the Raman signals associated with short periods of time appearance of these cells containing CNTs in the detected volume [Fig. 5(e)]. The clusters of CNTs inside the cells provided significant enhancement of the Raman signals, which increased the RFC capabilities to detect individual HeLa cells in the mesenteric structures.

To further understand the interaction and the developing kinetics of the CNTs after their administration in the lymphatics, Raman mapping analysis was performed by scanning the excitation laser beam across the mesentery tissue (Fig. 6 left) in two directions (X-Y mapping) at spatial step sizes of 1 \(\mu\)m of an area situated about 1 cm away from the injection site. The mapping images were built by using Horiba Jobin Yvon LabSpec 5.8 software, based on the XY variation of the 1568-cm\(^{-1}\) G band intensity. The lighter regions of the Raman images indicated higher concentrations of CNTs while the darker areas represent the lack of or lower concentration of CNTs (Fig. 6 right). The comparison of these 2-D Raman mapping images at 2, 12, 22, and 32 min after the CNT injection and the conventional transmission optical image (Fig. 6 left) indicated a good correlation of the size and shapes of Raman images with the lymphatic vessel size and margins (dashed lines in Fig. 6 right). These results suggest that the CNTs were initially distributed in the broad mesenteric area and then over time became more concentrated in a localized area of lymphatics (bright areas), followed by their further aspiration, collection, and traverse along lymph vessels leading to a decreased CNT concentration in the initial zone (darker spots). In particular, Raman mapping data taken 12 min after the initial injection indicated a very low concentration of CNTs in the interstitial tissue neighboring the lymphatics, while most of the CNT-specific Raman signal was derived preferentially from the lymph vessel. As time progressed further (22 and 32 min postdelivery), the CNTs were transported along the lymph vessels by the lymph flow indicated by an arrow in the optical image, leading to a sequential lower CNT concentration inside the lymphatic area that initially had a high CNT concentration. The Raman mapping data collected for CNTs were compared with the conventional fluorescent dye (FITC) kinetics (Fig. 7), injected intravenously in a rat’s tail vein at a concentration that was previously used for fluorescent microscopy and appeared in mesentery lymphatics approximately 1 h after injections. The fluorescent dye was introduced to monitor its kinetics (1 h after injection) into the lymphatic system and to compare its clearance rate with that obtained by Raman mapping for CNTs. The results demonstrate that fast Raman signal acquisition algorithms and their integration into 2-D Raman imaging of tissues by fast XY scanning provided a good correlation of the CNTs clearance time with the fluorescent dyes (FITC) when used for the same animal model. Therefore, despite the differences in FITC, CNT properties, and injection procedures, the CNTs were found to present a high mobility and ability to travel through the interstitial tissues, to enter into the lymph system, and to propagate through the lymphatics in a rough analogy to conventional dyes.

4 Discussion

The original experimental data presented in this paper indicate that Raman spectroscopy previously used to mostly study biological systems in stationary conditions in vivo or ex vivo after
The animals were sacrificed was successfully extended to dynamic *in vivo* studies of biological processes due to the unique properties of CNTs as Raman high-contrast agents and advances in fast Raman signal acquisition. As a result, for the first time, we showed the real-time Raman detection of CNTs in the bloodstream as well as the high ability of CNTs to distribute in tissues and accumulate and further traverse through the lymphatic systems. The distribution rate of CNTs in lymph vessels depends on the activity of these vessels, which in rat mesentery varies between a complete absence of lymph flow, low flow velocity in the range of 0.05 to 0.2 mm/s (as observed for the vessels that were analyzed in this study), and relatively high flow velocity up to 0.5 to 3 mm/s and even more in limited vessels. In addition to lymphatics, time-resolved RFC also demonstrated the capability for real-time detection of CNT pharmacokinetics *in vivo* in the blood circulation with relatively high flow velocity values of 3 to 5 mm/s. The travel time of individual CNTs through the detected volume under the parameters of our experiments was estimated to be around 3 to 5 ms, which is close to the temporal resolution of current RFC schematics (~7 ms). This makes it difficult to detect individual nanoparticles in fast blood flow. However, the current RFC schematic enables studying the CNT clearance rate when many nanoparticles are present in the bloodstream. According to our estimation, RFC, with the current threshold sensitivity, can be used for real-time monitoring of circulating CNTs with simultaneous presence of at least 60 individual CNTs (or their small clusters) in the laser focal volume. Nevertheless, this rough quantitative estimate requires further verifications. Further improvement may include new schematics for detection of flash Raman signals in the presence of a relatively stable background from tissue and blood, the use of optimized spectral range, linear laser beam geometry, and advancing the signal acquisition algorithm, which could approach the sensitivity detection threshold to a few individual CNTs in the detected volume.

The similar clearance kinetics obtained for CNTs and FITC may indicate that the behavior of the CNTs in various biological systems is likely driven by their small diameters rather than lengths, a fact which should indicate a “snaking” effect during their distribution and travel in blood, lymph, or other tissues. When CNTs penetrate and accumulate into cancer cells and assemble into clusters, a significant Raman signal enhancement provides the capabilities for *in vivo* detection of single cancer cells with their identification based on the specific spectral fingerprint of CNTs. Thus, this *in vivo* Raman flow cytometry technique can be considered as a new tool in biological research with many potential applications, including the study of CNT pharmacokinetics; visualization of lymphatics including lymph nodes (Raman lymphography); selective detection of circulating cancer cells, bacteria, or other biological systems targeted by CNTs; possibly functionalized with antibodies, DNA, or folate. The current schematic enables us to assess superficial microvessels with depth up to few (1 to 3) millimeters, which may be quite enough for many mentioned applications. Nevertheless, the use of the NIR spectral range and high contrast Raman agent (e.g., SERS tags) may further improve these parameters up to 1 to 2 cm.

The potential advantages of RFC with CNTs as contrast agents, compared to radioactive or fluorescent probes, is related to the relatively lower toxicity, rapid spectral CNT identification, and the selective targeting of cancer cells or infections. Nevertheless, additional studies are required to fully understand the potential toxic effects of the CNTs before their use in humans. Compared to the PA technique, RFC may provide high specificity of the corresponding scattering molecular spectra, which can be collected during cell movement through the detected volume. For spectral identification, the PA technique requires several laser wavelengths while Raman spectroscopy may operate with just one wavelength. Fluorescent measurements in flow cytometry are limited by the broad emission spectra of fluorescent labels. By contrast, Raman spectra exhibit relatively narrow spectral futures, and contain information on the chemical composition of the samples. On the other hand, the ability of CNTs to provide both strong Raman, fluorescent, PT and PA signals enables the possible integration of all these methods in one technical flow cytometry platform that could become universally applicable to objects with various absorption, fluorescent, and Raman scattering properties. In particular, the label-free nature of Raman and PT/PA spectroscopy using intrinsic cell molecular markers provides opportunities for *in vivo* non-invasive blood and lymph biochemistry analysis. The integration of RFC-based nanodiagnostics with PT molecular therapy and a NIR laser may provide RFC-guided selective nanophotothermal therapy with CNTs for treatment of lymphatic diseases, metastases (e.g. in the sentinel lymph nodes), circulating individual metastatic cells, or inhibition of pathological lymphatic networks around primary tumors to prevent the occurrence of metastasis.

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